Int. J. Dev. Biol. 63: 93-103 (2019)
https://doi.org/10.1387/ijdb.180355ks

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

Mitochondria and lipid metabolism in mammalian oocytes and early embryos

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ABSTRACT Mammalian oocytes and early cleavage-stage embryos are critically dependent on their ~100,000 mitochondria to develop from ovulation to compacted morula stage. They rely almost solely on oxidative phosphorylation of multiple intracellular substrates- namely pyruvate, fatty acids and glutamine- for production of ATP. Increasing evidence exists for the requirement of both fatty acids and pyruvate for mammalian developmental potential. Fatty acids are stored as neutral lipids in lipid droplets, which are liberated into the cytoplasm as free fatty acids and taken up into mitochondria for metabolism. Different mammalian species exhibit different amounts of stored and free lipid, while the types of lipid present tend to remain constant. It is thought that the amount of lipid contained in the oocytes of mammalian species reflects the extent of β-oxidation, but it is unclear why large differences are seen in lipid content. Maternal high fat diet or obesity causes negative intracellular effects such as the ER stress response, and oxidative mitochondrial and DNA damage. While some mechanisms have been established, it is still unclear exactly how high fat leads to compromised oocyte and embryo quality. It is proposed that healthy mammalian oocyte mitochondria require a balance of pyruvate and fatty acid oxidation in order to maintain a low level of otherwise damaging ROS production. This balance is disrupted in conditions of excess or insufficient substrate.

KEY WORDS: *mitochondria, lipid, metabolism, oocyte, embryo*

Introduction

Successful mammalian reproduction relies heavily on the quality of an oocyte, which is essential to ensure embryo development after fertilisation. Alongside predisposing genetic factors, an oocyte's quality is largely regulated by its maturation environment within the ovarian follicle, and its subsequent capacity for independent survival after ovulation. Within the follicle, a developing oocyte is surrounded by nutritive cumulus cells, which provide the oocyte with ATP and metabolic substrates such as pyruvate as a product of glycolysis. The surrounding follicular fluid also plays an important role in substrate provision, as it receives nutrients from the maternal bloodstream. Significantly, the composition of the follicular fluid, and therefore the oocyte itself is reflective of the mother's diet and health as a whole.

Once ovulated, mammalian oocytes rely almost solely on ATP from their ~100,000 mitochondria to survive until the compacted morula embryo begins glycolysis, prior to blastocyst implantation in the uterus (Dumollard *et al.,* 2008). Substrates for mammalian oocyte metabolism are much the same across different mammalian species, though their dependence on these substrates varies. Pyruvate, made available via the cumulus cells during oocyte follicular development, is known to be an important metabolic substrate in the oocytes of the majority of mammalian species (Dumollard *et al.,* 2008). Both human and mouse oocytes and early cleavage-stage embryos rely heavily on pyruvate oxidation as their main source of ATP. However, lipids are also an important source of energy, and may be the main substrate in porcine and bovine oocytes (Sturmey and Leese, 2003).

Abbreviations used in this paper: CARS, coherent anti-stokes raman scattering; CPT, carnitine palmitoyl transferase; DGAT, diacylglycerol acyltransferase; ER, endoplasmic reticulum; ETC, electron transport chain; FAD(H_2), flavin adenine dinucleotide (reduced); GV, germinal vesicle (immature) oocyte; H_2O_2, hydrogen peroxide; IMM, inner mitochondrial membrane; LD, lipid droplet; MCT, monocarboxylate transporter; MII, metaphase II (mature) oocyte; MMP, mitochondrial membrane potential; MtDNA, mitochondrial DNA; MUFA, mono-unsaturated fatty acid; NAD(H), nicotinamide adenine dinucleotide; $O_2^{\bullet-}$, superoxide anion; PDH, pyruvate dehydrogenase complex; PUFA, poly-unsaturated fatty acid; ROS, reactive oxygen species; SERCA, sarco-endoplasmic reticulum Ca^{2+} ATPase; TAGs. tri-acylglycerol; TCA, the citric acid cycle.

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Submitted: 18 October, 2019; *Accepted:* 26 November, 2018.

ISSN: Online 1696-3547, Print 0214-6282

Printed in Spain

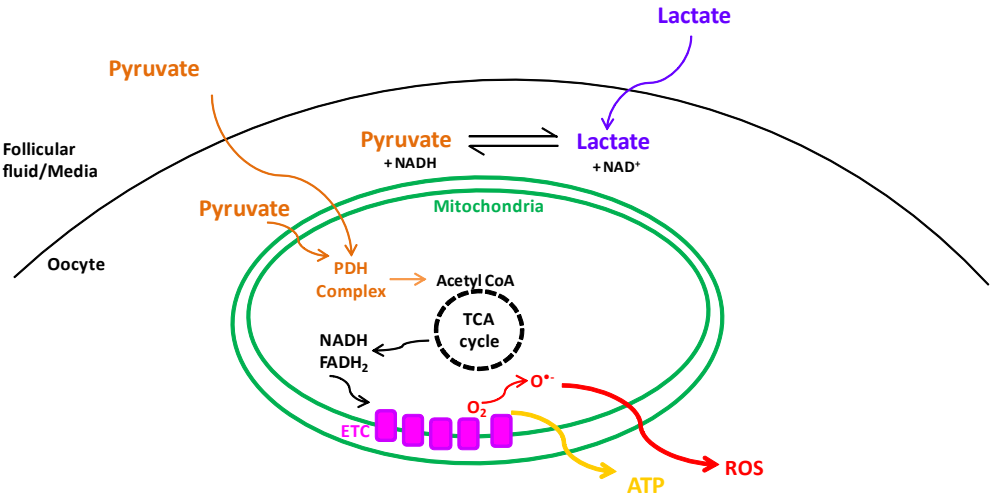

Fig. 1. Schematic of oocyte mitochondrial pyruvate metabolism. *Schematic showing pyruvate and lactate uptake into oocytes from the follicular fluid or surrounding media. Pyruvate is transported into mitochondria and oxidised by the pyruvate dehydrogenase (PDH) complex to acetyl CoA, which enters the citric acid (TCA) cycle of chemical reactions, reducing co-factors NADH and $FADH_2$. Electrons are transferred to a series of membrane-bound proteins in the electron transfer chain (ETC), ultimately reducing oxygen (O_2) to water in order to drive ATP production. Excessive pyruvate oxidation may cause electron leakage from the ETC and partial reduction of O_2 to superoxide ($O^{\bullet-}$) which generates damaging reactive oxygen species (ROS). Lactate and lactate-derived pyruvate are not taken up into mitochondria for ATP production, however it plays a role in redox balance through NADH generation. Cytosolic NADPH is able to reduce vital antioxidant GSH.*

In recent years there has been an increased appreciation of the importance of fatty acids as well as pyruvate oxidation in oocytes and early embryos. Nevertheless, excess substrate metabolism is widely known to cause sub-fertility and increased rates of miscarriage, particularly in prospective human mothers who adopt a high fat diet, and those that are clinically overweight or obese. Elevated levels of fatty acids within the oocyte, particularly saturated fatty acids, lead to decreased rates of fertilisation and embryo development, and can cause irreversible damage to foetal growth and offspring (Jungheim *et al.,* 2011a; Luzzo *et al.,* 2012).

Mitochondria and the quiet embryo hypothesis

Mitochondria meet the energy demands of the oocyte and cleavage stage embryo through uptake and oxidation of various substrates. This leads to generation of acetyl CoA in order to produce ATP via the Krebs/Citric Acid (TCA) cycle oxidation. Transfer of electrons via mitochondrial co-factors NAD(H) and $FAD(H_2)$ to the electron transport chain (ETC) creates a proton gradient across the inner mitochondrial membrane (IMM) generating the mitochondrial membrane potential (MMP). This drives oxidative phosphorylation, which generates ATP by the reduction of oxygen to water and the phosphorylation of ADP.

The many protein complexes comprising the ETC often allow leakage of electrons back into the mitochondrial matrix. Here, they are able to partially reduce molecular oxygen, producing what is known as the superoxide radical ($O_2^{\bullet-}$), a highly reactive molecule that is readily converted to hydrogen peroxide (H_2O_2), and other reactive oxygen species (ROS) (Quinlan *et al.,* 2013). Due to their highly oxidative nature, ROS cause oxidative damage, targeting proteins such as DNA including mitochondrial DNA (MtDNA). In general, basal ROS production can act as a natural regulatory mechanism for the cell, involved in general housekeeping and regulation of redox state (Quinlan *et al.,* 2013; Dumollard *et al.,* 2009). However, prolonged excess ROS production causes oxidative stress which is a precursor for apoptotic cell pathways, and thus ROS production must be kept to a minimum.

The mitochondrial substrates used by mammalian oocytes and early embryos depend on availability and differ between species. The primary sources of energy in all mammalian species appear to be pyruvate (derived from glucose), fatty acids, and amino acids. However, the use of each of these metabolic substrates by oocytes and cleavage-stage embryos of different species varies greatly.

The quiet embryo hypothesis applies to oocytes

Although almost solely relied upon to meet the energy demands of an egg and preimplantation embryo, there is a common misconception- at least in mammals- that oocyte mitochondria are inactive. Unlike somatic cells, animal oocyte mitochondria are generally more rounded than rod-shaped, and the IMM is smooth with few in-foldings, or cristae. This morphology and the finding that oocytes and embryos consume low amounts of oxygen suggests a lower level of metabolic activity (De Paula *et al.,* 2013; Dumollard *et al.,* 2009). However, instead of being 'inactive', it seems the mitochondrial activity of oocytes is simply 'turned down', allowing them to function only a minimal level. This phenomenon in oocytes probably also underlies the logic behind the 'quiet embryo hypothesis' or 'Goldilocks principle' described by Leese (2002; 2016), in which it is proposed that the viability of early embryos is dependent upon their ability to maintain an optimal level of metabolism (i.e. not too high or too low). It is believed that the oocyte and embryo maintain a low level of ETC activity in order to efficiently meet energy demands while minimising the production of oxidative ROS associated with oxidative phosphorylation. Even for large cells, the numbers of mitochondria present (>100,000) are considerably higher in oocytes than somatic cells, and they can occupy up to ~30% of the cytoplasmic volume (Dumollard *et al.,* 2009). Thus, it is likely that mitochondria are able to generate sufficient ATP without being 'overworked' and producing excess ROS.

Requirement for pyruvate oxidation in oocytes and early embryos

All mammalian embryos require pyruvate supplementation for successful development. Pyruvate is a derivative of glycolysis, a

process which is largely inactive in mouse and human oocytes and early preimplantation embryos. Thus, pyruvate is supplied to oocytes by cumulus cells within the ovary, and is also found in the surrounding follicular fluid. Pyruvate is readily taken up into the oocyte and the mitochondrial matrix via monocarboxylate transporters (MCTs) and is processed by the pyruvate dehydrogenase (PDH) complex to create acetyl CoA (see Fig. 1). The oxidation of two molecules of pyruvate generates ~30 molecules of ATP.

Pyruvate is the primary metabolic substrate used by mouse and human oocyte mitochondria, and may also be important in zygotic genome activation (Nagaraj *et al.,* 2017). However, it is provided at a rather low optimal concentration of ~0.2-0.3mM in *in vitro* culture media. An intermediate level of pyruvate oxidation is found to be optimal for development of human zygotes, consistent with the 'Goldilocks principle' (Turner *et al.,* 1994; Leese *et al.,* 2016). Pyruvate is found to directly scavenge hydrogen peroxide, thus starving mouse eggs of pyruvate affects their redox state, lowers ATP production, and inhibits egg activation at fertilisation, diminishing their developmental potential (Constantanopoulos and Barranger, 1984; Dumollard *et al.,* 2007, 2008). Thus there is a clear requirement for pyruvate metabolism for successful development. Conversion of lactate to pyruvate occurs under starved conditions (Dumollard *et al.,* 2007). Bovine embryos are able to reach blastocyst stage with lactate as their sole metabolic substrate (Takahashi and First, 1992), however, lactate-derived pyruvate is not used for ATP production in mouse oocytes, and the effects of pyruvate-starvation can only be avoided by the reintroduction of pyruvate (Dumollard *et al.,* 2007). This raises the question of the importance of lactate in murine oocytes, which may have a role in generating NADH, important for maintaining redox balance (Banrezes *et al.,* 2011).

Despite the obvious requirement for pyruvate in oocytes, excess pyruvate provision can have detrimental effects on oocyte and embryo development. Providing the cell with excess pyruvate fails to increase the amount of ATP produced, and in fact excess pyruvate decreases the rate of development to the blastocyst stage (Dumollard *et al.,* 2007). Interestingly, development to blastocyst can be restored by co-incubation with a ROS scavenger, suggesting that surplus pyruvate oxidation impairs development through increased ROS generation (Dumollard *et al.,* 2007). It is likely that increased mitochondrial metabolism of pyruvate generates ROS at the ETC (see Fig. 2), and that this outweighs pyruvate's ROS-scavenging properties.

Mammalian oocyte / early embryo lipid content

Lipid droplets in oocytes and early embryos

Unlike carbohydrates such as pyruvate, mammalian oocytes have endogenous stores of fatty acids in the form of lipid droplets (LDs). Free fatty acids within the blood serum enter the follicular fluid which surrounds the developing oocyte and their nutritive cumulus cells and are taken up into the oocyte. It is unknown to what extent fatty acids remain freely within the cytoplasm, or are taken up into the mitochondria for metabolism, however a large number are actively esterified to a glycerol molecule catalysed by the diacylglycerol acyltransferase (DGAT) enzyme and stored as neutral triacylglycerols (TAGs) in hydrophobic lipid vesicles.

LDs form at the ER, composing of a neutral TAG core and a single phospholipid layer, often with various protein inclusions (Fujimoto and Parton, 2011; Walther and Farese, 2012). They clearly have an important role in storage of energy substrate, but they also play a part in membrane maintenance, undoubtedly vital for the large increase in plasma membrane surface area seen in cleaving embryos; a 74% increase in plasma membrane is seen between the 2-cell and 4-cell embryo stages alone, indicating a substantially larger increase in later preimplantation stages (Pratt and George, 1989). A marked decrease in lipid content is noted as embryonic development continues (Romek *et al.,* 2009). Degradation of droplets tends to be via lipolysis of TAGs to fatty acyl coA by lipases at the LD surface, often in order to liberate fatty acids for mitochondrial metabolism. However, selective autophagy of LDs by liposomes (lipophagy) is also induced after fertilisation (Tsukamoto *et al.,* 2008).

Various techniques have been used historically to characterise LDs in eggs and embryos, including lipid stains, and fluorescent lipid dyes such as Nile Red and boron-dipyrromethene (BODIPY)

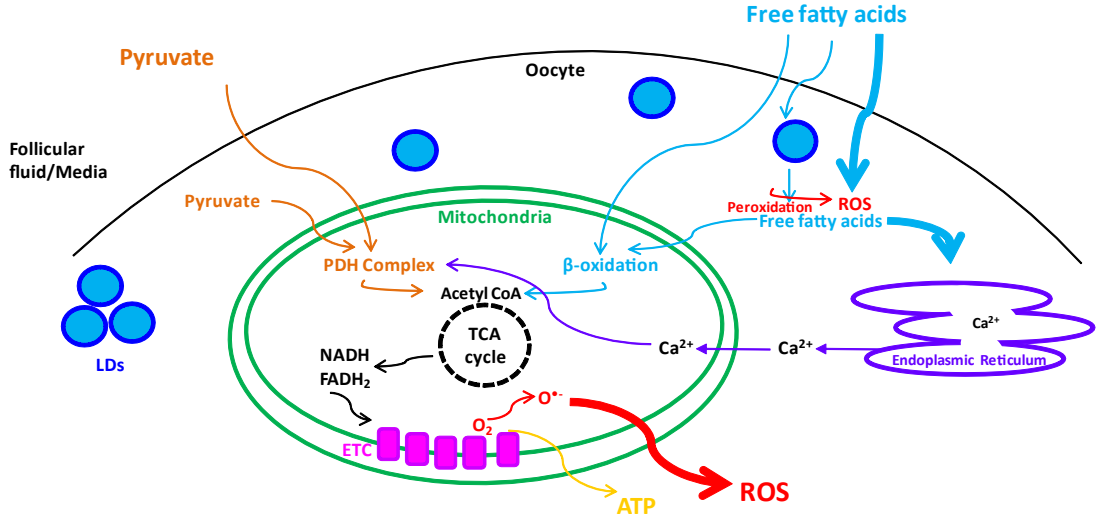

Fig. 2. Schematic of oocyte mitochondrial pyruvate and fatty acid metabolism. *Schematic showing pyruvate and free fatty acid uptake into oocytes from the follicular fluid or surrounding media. Free fatty acids are stored as neutral triacylglycerols (TAGs) in lipid droplets (LDs) or are taken up into mitochondria to undergo β-oxidation. In high fat conditions, such as obesity or high fat maternal diet, excess free fatty acids (depicted by a bold arrow) remain in the cytosol and may undergo lipid peroxidation, generating ROS. Free fatty acids may also directly affect the endoplasmic reticulum calcium (Ca^{2+}) store, causing ER stress and increasing the intracellular Ca^{2+} level. Ca^{2+} enters the mitochondria and causes increased ROS production via increased PDH activity. See Fig.3 for other details.*

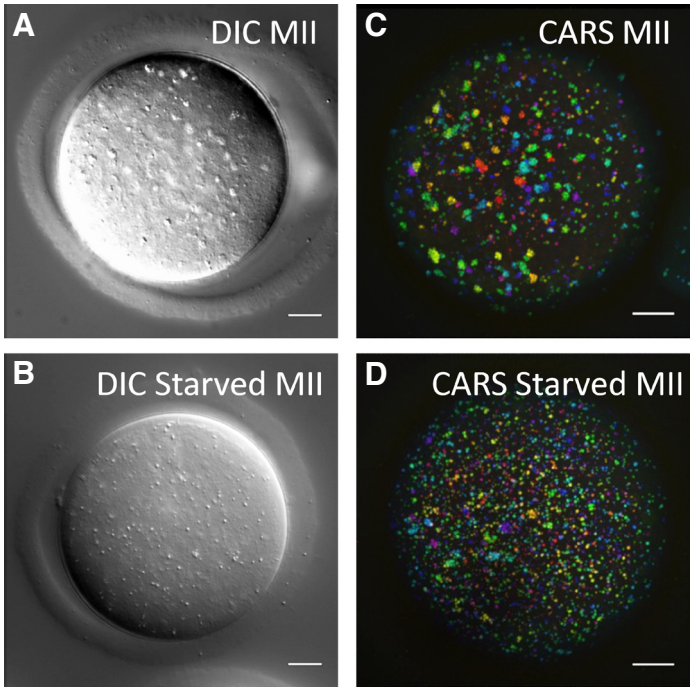

Fig. 3. DIC and CARS images of lipid droplets in control and carbohydrate-starved MII mouse eggs. (A,B) *Single-plane (approximately equatorial) DIC images.* **(C,D)** *Depth colour-coded images of CARS z-stacks at the lipid peak through the same eggs, showing lipid droplets in a representative* **(A,C)** *MII mouse egg (n=~70) and* **(B,D)** *carbohydrate-starved MII mouse egg (n=8). Scale bars represent 10 μm; colour bar shows depth colour-coding over 50 μm; the brightness of each colour is the maximum intensity at each corresponding z-plane.*

(Sturmey *et al.,* 2006; Yang *et al.,* 2010). While these stains aid visualisation of LDs, their use is invasive and they are often unspecific, staining lipid membranes of other organelles such as the Golgi apparatus or ER. More recently, coherent anti-Stokes Raman scattering (CARS) microscopy has been demonstrated as a non-invasive, chemically-specific method of LD detection (Bradley *et al.,* 2016). LDs were found to differ in size and number depending on the species, but also at different developmental stages (see Fig. 3). LDs of uniform size (~0.3μm) are seen in GV and MII stage mouse oocytes, and in earlier embryo stages, however fewer, larger LDs of differing sizes are found in cells of 8-cell to blastocyst stage embryos (Bradley *et al.,* 2016). It is likely that the LD distribution reflects the metabolic state of the oocyte or embryo, as a wide dispersion of LDs appears to be associated with increased β-oxidation in earlier developmental stages, while fusion of droplets coincides with glycolysis around morula stage.

Fatty acid composition of lipid droplets in oocytes

The actual fatty acid composition of oocyte LDs is dependent on the mother's diet or the surrounding media *in vitro*, determining the fatty acids available to the oocyte during ovarian development. *In vivo,* fatty acids are supplied to the oocyte from the follicular fluid via the surrounding cumulus cells. Dietary fatty acids in the bloodstream are responsible for the lipid profile of the follicular fluid and thus the oocyte (Valckx *et al.,* 2014). Although, there is evidence of the follicular cells acting as a buffer and mediating, to some extent, the fatty acids that are transported into the oocyte (Fouladi-Nashta *et al.,* 2009; Jungheim *et al.,* 2011b).

Multiple authors' analyses of the fatty acid content of mammalian eggs show that the greatest fraction of lipids in the majority of mammalian oocytes are saturated fatty acids, made up of a simple acyl chain containing no carbon-carbon double bond. Almost half of these consist of palmitic (16:0) and stearic (18:0) acids (McEvoy *et al.,* 2000; Haggarty *et al.,* 2006). Mono- unsaturated fatty acids (MUFAs) and polyunsaturates (PUFAs) make up ~30% and ~15%of total fatty acids, respectively. The most abundant MUFA is consistently oleic acid (18:1 n-9), while linoleic acid (18:2 n-6) and arachidonic acid (20:4 n-6) primarily constitute the PUFA component (McEvoy *et al.,* 2000; Haggarty *et al.,* 2006). Interestingly, PUFAs make up over double the total fatty acids in porcine oocytes than in ruminants, predominantly containing linoleic acid (McEvoy *et al.,* 2000).

Differences between the lipid content of oocytes from different mammalian species

Oocyte lipid content shows great disparity between differing mammalian species. Oocytes of domestic species such as dog, pig, cow and sheep contain a much higher lipid concentration than human or mouse. A darker oocyte cytoplasm reflects a higher lipid content; in particular, canine and porcine oocytes are densely packed with large lipid droplets, to the extent that their cytoplasm is famously opaque to light transmission (Prates *et al.,* 2012; McEvoy *et al.,* 2000; Romek *et al.,* 2009; Genicot *et al.,* 2005; Apparicio *et al.,* 2012). Oocytes from cows and sheep are also visually darker than those of mouse or human, which are transparent with no obvious LDs. Conventional lipid imaging methods confirm the presence of LDs in oocytes, showing fewer and smaller LDs in cow and sheep eggs than in pig.

Various techniques such as mass spectrometry, chemical extraction and fluorescent lipid staining have been used in an effort to quantify the actual lipid content of MII oocytes of each species. Together these give us a reliable estimation as to the lipid amount and allow us to make a comparison between oocytes of different mammals. While oocyte diameter varies between species, Table 1 shows the normalised amounts of fatty acids in ng per cubed μm of oocyte volume.

With the most abundant lipid, pig oocytes are estimated to contain ~25.7×10^{-5}ng fatty acids per μm^3 (McEvoy *et al.,* 2000; Sturmey and Leese 2003). The ovine oocyte content is nearer ~7.74×10^{-5}ng/μm^3 (McEvoy *et al.,* 2000; Coull *et al.,* 1998) while oocytes from cows contain ~5.69×10^{-5}ng/μm^3 fatty acids (Kim *et al.,* 2001; Ferguson and Leese, 1999), reflecting the more intermediate number of LDs visible. Mouse oocytes are found to contain even less lipid, ~2.05×10^{-5}ng measured per μm^3, consistent with their transparency and apparent lack of LDs under conventional microscopy methods (Loewenstein and Cohen, 1964). Very few investigations have provided data as to the lipid content of a human oocyte. The only noteworthy effort was made by Matorras *et al.,* in 1998. However, they determined human oocytes contain a surprisingly high ~48.1×10^{-5}ng fatty acid per μm^3. The significantly large standard deviations of these findings demonstrate the vast variability between women undergoing fertility treatments- often with undiagnosed genetic impairments (Delhanty, 2013)- and may even point towards variation between eggs of the same patient. The oocytes used in this study were also failed fertilisation oocytes, often thought to be of insufficient quality for developmental

viability, but also lacking the benefits of being freshly ovulated, considered 'old'.

The amount of lipid present in the oocyte could reflect the extent of β-oxidation in that cell to maintain its development throughout fertilisation and embryonic development. It is reasonable to consider that a higher lipid content reflects a greater reliance on fatty acids as a substrate for energy production. Pig oocytes for example, with their abundant LDs, maintain a high level of lipid metabolism, with fatty acids thought to be their primary source of ATP (McEvoy *et al.,* 2000; Ambruosi *et al.,* 2009; Sturmey *et al.,* 2006; Prates *et al.,* 2014). Porcine embryos are able to continue development with lipid alone as a substrate, but if they are provided with pyruvate they will oxidise it. Sheep and cow species are thought to have a moderate requirement for β-oxidation, reflecting their intermediate LD content (Gardner *et al.,* 1993; Ferguson and Leese, 2006). Darker bovine oocytes containing more lipid are found to have a higher development rate (Jeong *et al.,* 2009), but they also employ pyruvate oxidation to meet their metabolic demands. In contrast, mouse and human oocytes are thought to favour pyruvate as their major energy source and their resting lipid metabolism is thought to be low, possibly due to their low comparable lipid content (Harris *et al.,* 2009; Haggarty *et al.,* 2006; Downs *et al.,* 2009; Biggers *et al.,* 1967; Dumollard *et al.,* 2007; Conaghan *et al.,* 1993).

It is also possible that oocytes of some species have a greater capacity for lipid storage. The oocyte/embryonic expression pattern of DGAT1 differs between species. For example it is expressed at significantly higher levels in the pig oocyte prior to fertilisation, thus the rate of TAG synthesis and storage may be species-dependent (Jiang *et al.,* 2015). Although it is unclear if a higher capacity for lipid storage mirrors a higher capacity for lipid metabolism, it is likely this is the case. Measurement of the amount of TAG present reveals the relative amount of lipid stored in LDs. Pig oocytes are estimated to contain ~12.2×10^{-5}ng/μm^3 TAG, compared to ~2.2×10^{-5}ng/μm^3 TAG in ovine oocytes, and ~2.3×10^{-5}ng/μm^3 in cow (McEvoy *et al.,* 2000; Genicot *et al.,* 2005). These data are comparable to the values shown in Table 1, and may be used to determine the lipid storage capacity of the oocyte.

It is clear that the pig oocyte has a larger lipid reserve in the form of TAGs, possibly correlating with their higher metabolism of fatty acids. Knowledge of the TAG content, along with the phospholipid (PL) fraction of the total fatty acid content of oocytes, we are able to estimate the percentage of free fatty acids in the oocyte cytoplasm (McEvoy *et al.,* 2000). It appears that pig and cow oocytes contain a lower fraction of free fatty acids in their cytosol (27 and 28%, respectively) compared to sheep (44%), despite their higher overall lipid content. The reason for this is unclear. One possibility is that the fatty acid turnover is different between species. It is difficult to quantify the fatty acyl coA content which would enable assessment of the actual mitochondrial fatty acid uptake occurring. Measurement of oxygen consumption would be useful to determine this, and similar data in mouse oocytes would be useful for comparison. It is also possible that free fatty acid content may not correlate with total lipid content.

TABLE 1

TOTAL FATTY ACID LIPID CONTENT OF OOCYTES OF DIFFERENT MAMMALIAN SPECIES, NORMALISED TO VOLUME

Species	Oocyte Diameter (μm)	Oocyte Volume (μm^3)	Total Fatty Acid Content (ng)	Normalised Total Fatty Acid Content (ng/μm^3)
Pig	105	6.06×10^5	156	25.7×10^{-5}
Sheep	130ǂ	1.15×10^6	89	7.74×10^{-5}
Cow	125*	1.02×10^6	58	5.69×10^{-5}
Mouse	72	1.95×10^5	4	2.05×10^{-5}

Approximate total fatty acid lipid content (ng/μm^3) of oocytes of the most-studied mammalian species. Data collated from: McEvoy *et al.,* 2000; Sturmey and Leese 2003; Loewenstein and Cohen, 1964; Ferguson and Leese, 1999; Kim *et al.,* 2001; Coull *et al.,* 1998; Fair *et al.,* 1995; Shirazi and Sadeghi, 2007; Griffin *et al.,* 2006.

ǂ Average between 110-150μm (Shirazi and Sadeghi, 2007)

*Average between 110-140μm (Fair *et al.,* 1995; McEvoy *et al.,* 2000)

Litter size has also been a proposed explanation, as dogs and pigs have larger litters than most domestic mammalian species, however this does not explain the vast difference between them and mice, which also produce sizable litters. Cows and sheep are both ruminants, but it is unclear why this would set them apart from other species by having an intermediate level of lipid.

Requirement for fatty acids in oocytes and early embryos

Fatty acids are carbon-dense, their aliphatic chains containing between 4 and 28 carbons, meaning they are energy-rich and can generate ~106 ATP per molecule. Carnitine-bound fatty acyl coA are transported via mitochondrial membrane-bound carnitine palmitoyl transferase (CPTI and II) proteins into the mitochondrial matrix in order to undergo β-oxidation to acetyl CoA. The requirement for fatty acids in mouse eggs has been demonstrated, despite their apparent reliance on pyruvate as a substrate. Inhibition of mitochondrial fatty acid uptake, via the use of CPTI inhibitor etomoxir, hinders mouse oocyte maturation and reduces the rate of blastocyst development (Dunning *et al.,* 2010, 2011; Downs *et al.,* 2009). Meanwhile, inclusion of L-carnitine in the *in vitro* culture media to promote fatty acid β-oxidation improves rates of both oocyte maturation and embryo development after fertilisation (Dunning *et al.,* 2012). Successful *in vitro* cell culture benefits from the addition of bovine serum albumin (BSA) to the culture media, providing the cell with multiple fatty acids (Downs *et al.,* 2009; Ferguson and Leese, 1999). Thus, it is clear that fatty acid oxidation plays some role in maintaining mouse oocyte viability. Underfeeding and lower body weight also presents lower blastocyst rates and

It is not currently understood why lipid content and metabolism in oocytes and embryos of different mammalian species varies so greatly. Suggested explanations include a prolonged time to implantation in species with a higher lipid reserve. While rates of development to blastocyst are relatively similar, canine embryos implant into the mother's uterus ~day 22 post-fertilisation, porcine embryos ~ 11days post-fertilisation, while human blastocysts implant ~day 6 and mice ~ 4 days (Concannon *et al.,* 2001; Prates *et al.,* 2014; Schatten and Constantinescu, 2008). Porcine blastocysts also maintain their inner cell mass for ~6-7 days post-implantation, compared to ~1-3 days in mouse and human embryos (Oestrup *et al.,* 2009), meaning pig embryos may require a larger reserve of metabolic substrates to survive for a longer period of time. However, this correlation between lipid content and time to implant does not fit with bovine embryos which do not implant until ~day 30 post-fertilisation, and sheep blastocysts which take ~21-22 days (Lee and DeMayo, 2004; Giminez and Rodning, 2007). Blastocysts from goat species do not implant until ~52 days post-fertilisation but their lipid content is thought to be similar to that of sheep (Giminez and Rodning, 2007).

increased apoptosis in blastocyst cells, suggesting a low threshold requirement for sufficient dietary fatty acids for successful oocyte development (Grazul-Bilska *et al.,* 2012; Kubandova *et al.,* 2014).

Negative effects of high fat on oocytes and embryos

Although there is a clear requirement for β-oxidation to aid oocyte development, it is well-recognised that high fat conditions are detrimental to oocyte and embryo quality. Despite the efforts of the ovary and follicular cells to mediate fatty acid transport from the blood into the oocyte, the maternal diet and body composition has a clear influence over the oocyte's lipid content and thus their quality and overall developmental potential. A higher amount of body fat or even simply a diet high in fat can negatively alter the ability of an oocyte to fertilise and continue embryonic development to birth. Systemic changes such as high blood insulin and inflammation can result from high levels of free fatty acids within the blood, directly affecting the ovarian environment and subsequently the oocyte (Robker *et al.,* 2009).

There are notably higher concentrations of free fatty acids in the follicular fluid of obese mothers, the lipid content and infertility rates positively correlating with increasing BMI (Valckx *et al.,* 2014). The follicular fluid lipid profile can be used as a predictor of quality, higher concentrations of saturated fatty acids- namely palmitic and stearic acids- resulting in failure to cleave after fertilisation (O'Gorman *et al.,* 2013). Elevated free fatty acids in the culture media during bovine *in vitro* maturation leads to apoptotic follicles. This results in fewer matured oocytes with poor mitochondrial morphology and blastocyst defects, including reduced cell number and apoptotic mechanisms (van Hoeck *et al.,* 2011). The cumulus cells of high fat diet oocytes are also shown to have a higher incidence of abnormal mitochondria, suggesting even further upstream implications (Luzzo *et al.,* 2012). Finally, it has been found that oocytes from women with higher concentrations of free fatty acids within their follicular fluid and serum show poor overall morphology, and higher cases of endometriosis are also noted, suggesting lower implantation rates even if fertilisation is successful (Jungheim *et al.,* 2011b). Embryos from obese mothers also demonstrate meiotic aneuploidy often causing premature embryonic loss, but also leading to foetal growth retardation and developmental brain abnormalities, putting offspring at risk of miscarriage or long-term behavioural and cognitive disorders (Luzzo *et al.,* 2012; Jungheim *et al.,* 2011a).

The effects of a high fat environment are mostly mediated at the oocyte level. Although the detrimental effects of maternal high fat diets and obesity on fertilisation, embryo implantation and foetal development are likely to be multifactorial, follicular exposure of developing oocytes to increased levels of fatty acids causes changes at a cellular level that predisposes the egg to a poorer survival prognosis. It has been convincingly shown that IVF outcomes for obese patients can be improved if eggs from a normal weight donor are used (Luke *et al.,* 2011; Jungheim *et al.,* 2013b). Similarly, donor eggs from mothers on a high fat diet suffer the same developmental defects when implanted into a surrogate of normal weight (Luzzo *et al.,* 2012). Pronuclear transfer from an oocyte from a high fat environment to an oocyte from a low fat environment also improves their success rate, suggesting a healthier cytoplasmic environment improves survival. Resumption of a healthy diet is not sufficient to reverse the effects obesity has on developing oocytes, and a gestational high fat diet will also affect the developing offspring (Reynolds *et al.,* 2016; Sasson *et al.,* 2015). Weight loss improves both natural and assisted conception in obese patients, and reduces the risk of developmental deficits in the offspring (Clark *et al.,* 1998).

Under conditions of high levels of intracellular lipid, free fatty acids undergo lipid peroxidation within the cytoplasm, producing excess ROS and toxic lipid peroxides and depleting protective glutathione levels, in a process known as lipotoxicity (Igosheva *et al.,* 2010) (see Fig. 2). This is known to occur not only within the oocyte but within other ovarian cells which may have further influence over the development of the oocyte (Wu *et al.,* 2010, 2012a). Excess ROS are known to be highly cytotoxic, and are harmful to mitochondrial and nuclear DNA, leading to the oocyte mitochondrial and spindle abnormalities often associated with obesity (Turner and Robker, 2014; Luzzo *et al.,* 2012). Many organelles and thus cellular processes are impaired as a result, such as structural alterations to the mitochondria and ER, and apoptotic pathways are initiated (Wu *et al.,* 2010; Engin, 2017).

An important consequence of lipotoxicity is the disruption of the ER, known as the ER stress response. This response is characterised by ER dysfunction after oxidative damage and loss of calcium through channels from the internal calcium store into the cytosol. This disrupts the vital calcium homeostasis of the cell and can lead to impaired protein folding and ultimately apoptotic mechanisms. It is likely that a large release of calcium from the ER results in excessive calcium entering the mitochondria and disrupting their function (Malhotra and Kaufman 2007; Wu *et al.,* 2015, 2012b) (see Fig. 2). It is also apparent that there is increased interaction between ER and mitochondria in oocytes from obese mice, which likely is responsible for this increased calcium exposure (Zhao *et al.,* 2017).

The most common model for ER stress is to expose cells to a high concentration of palmitic acid, mimicking exposure of high concentrations of free fatty acids as would be seen in overweight or obese mothers, and known to cause a full ER stress response. It is possible that the sarco/endoplasmic reticulum calcium ATPase (SERCA) pumps on the ER responsible for pumping calcium back into the store are directly affected by free fatty acids, as SERCA pump inhibitor thapsigargin creates the same ER stress response as high doses of palmitic acid (Wu *et al.,* 2012b, Borradaile *et al.*2006). Borradaile *et al.* (2006) note the incorporation of palmitic acid into the ER membrane, disrupting its structure and integrity, possibly compromising pumping of calcium back into the ER store. Particular negative responses to excess fatty acid exposure, such as embryonic arrest and various mitochondrial defects can be rescued by treating embryos with antioxidants, suggesting that lipid peroxidation and excess ROS production play a major role (Nonogaki *et al.,* 1994; Boots *et al.,* 2016). However, effects of palmitic acid cannot be reversed by addition of antioxidants, suggesting that palmitic acid induces embryonic arrest via different mechanisms (Nonogaki *et al.,* 1994). The calcium chelator BAPTA can be used to reduce cell death in cells exposed to high palmitic acid concentrations (Zhang *et al.,* 2012).

It is also likely that an increased availability of fatty acids leads to an increase in their mitochondrial oxidation (see Fig. 2). Igosheva *et al.,* (2010) find that mothers on a high fat diet have an increased oviductal leptin, which stimulates fatty acid oxidation gene upregulation through activation of the nuclear PPARγ receptors. The redox state of the cell is thought to become more

reduced, as seen by a decrease in mitochondrial FAD autofluorescence with exposure to high concentrations of palmitic acid, suggesting an increase in TCA cycle activity (Sutton-McDowall *et al.,* 2016). Increased TCA and therefore ETC activity has the potential to further increase the production of ROS, especially in cases where oxidative phosphorylation cannot keep up with the extent of substrate oxidation, and electron leakage ensues. In turn this also has the potential to increase oxidation of free fatty acids in the cytosol, as incomplete β-oxidation of fatty acids results in shorter acyl chains susceptible to cytoplasmic peroxidation (Muoio and Neufer 2013).

Interestingly, fatty acid uptake facilitators L-carnitine and AICAR rescue mitochondria from lipotoxic stress in multiple cell systems, while fatty acid uptake inhibition increases cell death (Oyanagi *et al.,* 2011; Borradaile *et al.,* 2006). Also, forced-lipophagy aids survival of cleavage-stage embryos exposed to excess lipid (Tatsumi *et al.,* 2018). These suggest an increase in the metabolism of fatty acids is a protective mechanism in an attempt to remove free fatty acids from possible oxidation in the cytoplasm. The cell makes an effort to adapt to increased stress and is initially pro-survival in its responses, but ultimately apoptosis will result if stress is prolonged (Rutkowski *et al.,* 2006). Wu *et al.,* (2012b) found that L-carnitine did not rescue oocyte mitochondria treated with high doses of palmitic acid (400μM), but it could be argued that the unphysiological concentrations used are too high to cause reversible effects. The total fatty acids in follicular fluid from obese patients is estimated ~315.53 ± 82.68μM, while palmitic acid makes up ~70.3 ± 17.1μM, thus 400μM may be an unphysiological exposure (Valckx *et al.,* 2014).

There are conflicting results as to the effects of a high fat environment on mitochondrial membrane potential (MMP). Wu *et al.,* (2010) noted that oocytes of mice fed a high fat diet displayed a decreased MMP, as did those incubated in lipotoxic conditions of high palmitic acid concentrations (Wu 2012b). This is indicative of a lower mitochondrial metabolic ETC activity, or increased uncoupling, and would ultimately result in decreased generation of ATP often seen in lipotoxic stress. It is likely that this response is due to ER stress rather than causing it, as effects can be rescued by ER stress inhibitor salubrinal, and this suggests that mitochondria are damaged rather than inactivated by increased calcium and excess ROS exposure. Conversely, Igosheva *et al.,* (2010) saw a marked increase in MMP in oocytes and zygotes from mothers on a high fat diet, describing an increase in general mitochondrial oxidation. Sutton-McDowell *et al.,* (2016) found bovine oocytes exposed to a mixture of free fatty acids showed no difference in MMP.

It is not clear why different studies have reported these differing effects of excess fatty acids on MMP. It could be that there are multiple levels of lipotoxicity, with various mechanisms triggering different pathways, ultimately having differing effects on mitochondrial activity. In *in vivo* high fat conditions, it is likely that various systemic effects also come into play, whilst buffering systems in place mediate oocyte exposure, providing cytoprotective mechanisms to a certain extent. *In vitro,* it is possible that overexposure of oocytes to high doses of fatty acids brings about a stress response, leading to a dissipation of MMP (Wu *et al.,* 2012b). There may also be discrepancies in results due to variations in MMP measurement techniques, meaning the true effect of high fat on MMP is yet to be elucidated. It is also likely there are species differences in the way oocytes are able to adapt to changes in their lipid environment, as pig oocytes clearly can tolerate a higher lipid concentration than mouse oocytes. It is interesting that an increase in ROS production is seen despite an apparent decrease in mitochondrial activity, as usually a reduced MMP coincides with lowered ROS generation (Korshunov *et al.,* 1997).

The types of fatty acid oocytes are exposed to during development also influence their quality and developmental potential. Unsaturated fatty acids accumulating in the cytosol are more efficiently esterified into TAGs than saturated fatty acids, MUFAs such as oleic acid being shown to promote LD formation as they are preferentially incorporated by DGAT for TAG formation (Nolan and Larter 2009; Aardema *et al.,* 2011). Higher-than-normal levels of saturated fatty acids such as palmitic acid are more likely to undergo cytosolic peroxidation and initiate apoptotic pathways, coinciding with reduced fertilisation rates and defective development (Jungheim *et al.,* 2011a; Shaaker *et al.,* 2012; Nonogaki *et al.,* 1994). Oleic acid is even found to rescue the negative phenotypes associated with palmitic acid, possibly due to its role in aiding formation and storage of TAGs in LDs (Aardema *et al.,* 2011). Low quality bovine oocytes and human oocytes that have failed to fertilise after IVF display increased concentrations of saturated fatty acids, namely stearic acid (Kim *et al.,* 2001; Haggarty *et al.,* 2006). Alternatively, unsaturated fatty acids- specifically n-6 fatty acids- improve embryo morphology and blastocyst rates (Jungheim *et al.,* 2013a; Hammiche *et al.,* 2011).

Hypothesis of a 'balanced diet'

It is clear that mitochondrial metabolism of both pyruvate and fatty acids is crucial for mammalian oocyte and embryo development. However, it is apparent that excess mitochondrial oxidation of either pyruvate or fatty acids is detrimental to developmental potential, due to the generation of excess ROS. It is also highly likely that the abundant oocyte and early embryo mitochondria are active but that their activity is 'turned down' in order to minimise ROS production. We propose that the quiet embryo hypothesis and Goldilocks principle are supplemented with a 'balanced diet' hypothesis. This specifically suggests that low mitochondrial activity is maintained by a balance of oxidation of both pyruvate and fatty acids by mitochondria in order to keep the levels of ATP and ROS generation 'just right' for optimal development. The idea of a balance between pyruvate and fatty oxidation is similar to that of the Randle cycle, which describes a balance between β-oxidation and glycolysis in somatic cells (Hue and Taegtmeyer, 2009; Randle *et al.,* 1963). Since glycolysis is inactive in ovulated oocytes and cleavage stage embryos the equivalent would be β-oxidation and pyruvate oxidation. In addition, the Randle cycle is used to explain fuel selection for maintaining ATP, but in oocytes this is less the issue. It may be more about a mechanism to maintain a low level of ROS production. We would expect that any manipulations that shift the oocyte away from a balance point, and hence shift to predominantly fatty acid or pyruvate use, would excessively increase ROS production, with negative consequences. Oocytes and other cell systems treated with β-oxidation inhibitor etomoxir show increased PDH complex activity and shift to carbohydrate metabolism in response to a loss of fatty acids as a metabolic substrate (Bryson *et al.,* 1996; Sturmey and Leese, 2008; Hewitson and Leese, 1993). Interestingly, inhibition of β-oxidation also causes

increased ROS production, suggesting an over-compensatory shift in mitochondrial activity (Merrill *et al.,* 2002). It is possible that the inhibitory effects of etomoxir on mouse embryo development are mediated by increased PDH -generated ROS production.

Species differences

There is an interesting disparity between some mammalian species in the amount of pyruvate required for optimal development that mirrors the discrepancies seen in lipid content. This is apparent when considering the inclusion of metabolites in different *in vitro* culture media suited for oocytes and cleavage-stage embryos of different species. For example, pyruvate is included in mouse or human oocyte culture media at ~0.2-0.3mM, a physiological concentration which is surprisingly low for cells which supposedly rely primarily on this as an energy source. It could be that mouse and human oocytes have more effective mitochondrial pyruvate transport and thus have a high pyruvate turnover. However, if the low cellular lipid content is taken into account, the levels of pyruvate and fatty acids might both be considered to be relatively similar and low. In culture of pig oocytes, pyruvate is included nearer to 2-5mM, a concentration that seems high unless considering that it may reflect the high lipid content of porcine eggs and embryos (Dumollard *et al.,* 2009). If the pyruvate concentration in mouse/human oocyte culture media was increased to nearer the concentration in pig oocyte culture media, survival rates would be compromised, as would porcine rates if concentrations were lowered. Therefore, the pyruvate requirement for oocytes and cleavage-stage embryos appears to reflect the level of β-oxidation occurring, possibly in balance to reduce the production of excess ROS that would result if either substrate were relied upon as the sole source of ATP. This would explain why lipid or pyruvate levels normally toxic to mouse or human oocytes are entirely reasonable for porcine oocyte and embryo development.

It is likely that pig oocytes, able to survive to blastocyst with little or no pyruvate supply, are able to mobilise more lipid in compensation, their larger lipid stores meaning they will survive for longer than e.g. a mouse oocyte with less available lipid. This conclusion can be drawn from data from Bradley *et al.,* (2016), where carbohydrate-starvation of mouse oocytes lead to a wide dispersion of lipid droplets (see Fig. 3), likely in a bid to mobilise more fatty acids for mitochondrial uptake in the absence of pyruvate. However, in mouse embryos this is not compatible with long-term survival, likely due to their smaller lipid reserves.

Optimal metabolic thresholds

It appears there are optimal thresholds of mitochondrial pyruvate and fatty acid metabolism the oocyte requires in order to maintain its quality and healthy development, consistent with the 'Goldilocks principle'. In the healthy egg or early embryo, a balance of metabolic pyruvate and fatty acid oxidation is maintained. In the absence of either substrate, we propose there is increased uptake of the other. As high fat conditions promote increased β-oxidation, it is likely that pyruvate metabolism is subdued in turn. It is known that high fat diet inactivates PDH complex activity in multiple tissues through activation of PDH kinase, possibly through the increase of acetyl coA and NADH generation (Rinnankoski-Tuikka *et al.,* 2013; Orfali *et al.,* 1993). Increased acetyl coA production as a result of excess pyruvate oxidation is also said to inhibit enzymes in the β-oxidation pathway (Jaswal *et al.,* 2011).

Concluding remarks

It is clear that mitochondrial oxidation of both fatty acids and pyruvate plays an essential role in mammalian oocyte and embryo development. However, an excess of either such as in a high fat diet has detrimental consequences for fertility and conception. Here we propose that a balance of β-oxidation with pyruvate metabolism is a conserved mechanism of maintaining a low toxicity mitochondrial metabolism whilst generating sufficient energy for continued development. An excess oxidation of either could result in generation of excess ROS. Species differences in lipid content and the extent to which they use fatty acids as a metabolic substrate are marked. Although it is unclear as to why this might be, the same principle of a substrate balance seems to apply, as species whose oocytes contain a larger fat reserve also rely on higher concentrations of pyruvate for survival. Species with oocytes with a higher lipid content may not have a higher rate of β-oxidation, and fatty acids may have the same contribution to ATP production as oocytes with a lower natural lipid content.

Excess production of ROS causes damage to cellular proteins and DNA, including MtDNA. Damage to nuclear and MtDNA often results in aneuploidy and decreased rates of fertilisation and development due to decreased mitochondrial copy number and compromised ETC function. Mutations in MtDNA are particularly problematic due to their presentation in offspring as disabling and often life-threatening maternally-inherited mitochondrial diseases (Burgstaller *et al.,* 2015; Srirattana and St John 2018). It is therefore conceivable that a balance in substrate oxidation is a conserved mechanism vital for preservation of not only current oocyte or embryo viability, but the health of future generations.

Acknowledgments

Work in our laboratory is support by the BBSRC. We thank Paola Borri, Wolfgang Langbein and Iestyn Pope for discussions and help with preparation of images in Fig. 3.

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